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Article

Dose-dependence of radiotherapy-induced changes in serum levels of choline-containing phospholipids; the importance of lower doses delivered to large volumes of normal tissues.

Karol Jelonek [1,*], Aleksandra Krzywon [1], Katarzyna Papaj [2], Pawel Polanowski [1], Krzysztof Szczepanik [1], Krzysztof Skladowski [1] and Piotr Widlak [1]

[1] Maria Sklodowska-Curie National Research Institute of Oncology, Gliwice Branch, Wybrzeze Armii Krajowej 15, 44-102 Gliwice, Poland; Aleksandra.Krzywon@io.gliwice.pl (A.K.); Pawel.Polanowski@io.gliwice.pl (P.P.); Krzysztof.Szczepanik@io.gliwice.pl (K.Sz.); Krzysztof.Skladowski@io.gliwice.pl (K.Sk.); Piotr.Widlak@io.gliwice.pl (P.W.)

[2] Biotechnology Centre, Silesian University of Technology, Krzywoustego 8, 44-100 Gliwice, Poland; Katarzyna.Papaj@polsl.pl (K.P.)

* Correspondence: Karol.Jelonek@io.gliwice.pl; Tel.: +48-32-278-9627

Simple Summary: Radiotherapy-induced changes in serum levels of choline-containing phospholipids depended on the dose and volume of irradiated tissues. Higher doses and volumes were associated with reduced levels of (lyso)phosphatidylcholines and increased levels of sphingomyelins. Strong effects of large volumes of normal tissues irradiated with clinically low doses (i.e., accumulated dose 20 Gy) on the systemic response to IMRT were observed in patients treated due to head and neck cancer.

Abstract: Conformal radiotherapy is a primary treatment in head and neck cancer, which putative adverse effects depend on relatively low doses of radiation delivered to increased volumes of normal tissues. Systemic effects of such treatment include radiation-induced changes in serum lipid profile, yet dose- and volume-dependence of these changes remain to be established. Here we analyzed levels of choline-containing phospholipids in serum samples collected consecutively during the radiotherapy used as the only treatment modality. The LC-MS approach applied in the study enabled the detection and quantitation of 151 phospholipids, including (lyso)phosphatidylcholines and sphingomyelins. No statistically significant differences were found in the pre-treatment samples from patients with different location and stage of cancer. To compensate for potential differences between schemes of radiotherapy the biologically effective doses were calculated and used in the search of correlations with specific lipid levels. We found that the levels of several phospholipids depended on the maximum dose delivered to the gross tumor volume and total radiation energy absorbed by the patient's body. Increased doses correlated with increased levels of sphingomyelins and reduced levels of phosphatidylcholines. Noteworthy, serum phospholipid levels were associated mainly with volumes of normal tissues irradiated with relatively low doses (i.e., total accumulated dose 20 Gy), which indicated the importance of such effects on the systemic response of the patient's organism to IMRT.

Keywords: dose-response; head and neck cancer; mass spectrometry; lipidomics; radiotherapy; radiation response

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1. Introduction

Head and neck squamous cell carcinoma (HNSCC) involves different squamous cell carcinomas located in the larynx, pharynx, oral cavity, and tongue, i.e., organs that play crucial roles in respiratory, nutritional, social, and communicative functions. HNSCC is the sixth most common malignancy and accounts for approximately 6% of all cancer cases worldwide [1]. The primary treatment for HNSCC is surgery and/or radiotherapy (RT) applied alone or in combination with chemotherapy [2]. Currently, RT is delivered using techniques of conformal radiation therapy, including intensity-modulated radiotherapy (IMRT), where a high dose better conforms to the tumor shape, enabling a reduction of the dose delivered to adjacent critical organs [3]. However, a potential drawback of IMRT is an exposure of a large volume of normal tissues to low/medium doses. Hence, this approach may still increase the risk of undesirable adverse effects in normal tissues, which may not only reduce the patients' quality of life [4] but also lead to unplanned therapy interruptions [5]. Importantly, in HNSCC a one-day gap in RT could decrease the local control rate by 1.4%, while a gap of one week is correlated with an absolute reduction in local control rates of 10-12% [6]. Therefore, hypothetical molecular markers for the monitoring of individual response to radiation might significantly improve the quality of HNSCC treatment.

The response to RT was already observed as the systemic effect in body fluids at all "omics" levels, including genomics, proteomics, and metabolomics [7-12]. Lipidomics is one of the most complex areas of metabolomics and it is dealing with dynamic changes of cellular lipids and their derivatives [13]. Glycerophospholipids (GP) and sphingolipids (SL) that include a choline group have essential structural and signaling functions [14,15]. These classes of lipids were already shown to be affected in response to ionizing radiation, both local body RT in humans [16] and whole body irradiation in rodents [17]. Our previous study on lipid mass profiling documented that serum levels of different phospholipids were affected in samples of HNSCC patients treated with IMRT using the continuous accelerated scheme CAIR. Moreover, the analysis based on the comparison of pre-RT and post-RT samples revealed that changes in levels of several compounds (putatively choline-containing phospholipids) were associated not only with a maximum dose delivered to the tumor target but also with a volume of tissues irradiated with lower doses [18]. Here we plan to further extend this observation, aiming to identify specific choline-containing lipids affected by radiation during RT and to reveal the dependence of RT-related effects on a radiation dose and volume of irradiated tissues using a series of serum samples collected through the whole treatment.

2. Results

The analysis of serum lipidome was performed in a group of HNSCC patients treated with IMRT alone according to different treatment plans (Table 1). Using the liquid chromatography coupled with mass spectrometry (LC-MS) approach, based on the compound's retention times and mass/charge (m/z) values, we detected and quantitated 151 choline-containing lipids (or their isomer groups), including 81 phosphatidylcholines (PCs), 12 lysophosphatidylcholines (LPCs), and 58 sphingomyelins (SMs). Further fragmentation patterns using tandem mass spectrometry additionally confirmed the specific identification of 43 lipids (lipid names were used in this case instead of lipid class and m/z identifier). The analysis was performed in serum samples collected consecutively every week of the treatment (approximately 5 intra-RT samples per patient, 249 samples in total). The involved group of patients was rather heterogeneous concerning cancer stage and location (Table 1). Therefore, to estimate the potential effect of these confounding factors, relevant differences in lipid profiles were addressed in pre-RT serum samples. A few lipids were found that showed different levels in sera of patients with less and more advanced cancer. When compared patients with T1-T2 vs. T3-T4 tumor size and patients with N0 vs. N1-3 local lymph node status, there were 9 and 14 compounds that showed differences at the significance level $p<0.05$, respectively, yet none of them remained statistically significant if the correction against multiple testing was applied. Similarly, no statistically

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significant differences were observed when pre-RT serum samples were compared for patients with two major cancer locations (i.e., larynx and pharynx). Therefore, we concluded that RT-related changes observed in analyzed samples should be primarily affected by doses of radiation and/or volume of irradiated tissues.

Table 1. Characteristics of the patient group.

Samples	within-RT	pre-RT
Number of patients	45	53
Number of samples	249	53
Age [years] (median)	46–77 (62)	40-75 (57)
Sex: male/female	34/11	40/13
Tumor localization	n	n
Larynx	31	27
Pharynx	11	26
Oral and nasal cavity	3	-
Tumor size (T)	n	n
T1	5	5
T2	29	22
T3	8	16
T4	3	10
Lymph node status (N)	n	n
N0	39	27
N1	3	4
N2	3	20
N3	0	2

IMRT scheme		n
1	1.8 Gy/fraction; 38-40 fractions ; 68.4-72 Gy total dose	23
2	2 Gy/fraction; 30-35; fractions; 60-70 Gy total dose	5
3	2.2 Gy/fraction; 30 fractions; 66 Gy total dose	5
4	2.25 Gy/fraction; 27 fractions; 60.75 Gy total dose	1
5	2.5 Gy/fraction; 24-25 fractions; 60-62.5 Gy total dose	7
6	3 Gy/fraction; 17 fractions; 51 Gy total dose	4

In general, we searched for the association between RT-induced changes in levels of specific phospholipids and radiation dose (or volume of irradiated tissues). However, the study included patients treated with different irradiation schemes with putatively different biological effects. Therefore, to compensate for this effect, biologically effective doses (BED) were calculated in each case and used instead of "physical" doses. In the first step, we searched for lipids which serum levels were associated with a maximum GTV (gross tumor volume) dose accumulated at a given time point. We found 16 lipids (2 LPCs, 11 PCs and 3 SMs) that showed statistically significant correlations (p-value < 0.05 and $|r| > 0.3$) with a maximum GTV dose (examples of identified compounds are presented in Table 2). Further, we searched for the association between levels of lipids and total radiation energy absorbed by the patient's body. This parameter was estimated from the individual dose-volume histogram (DVH) by calculating its integral over a dose or area under the curve (radiation dose multiplied by mass approximated from a volume of irradiated tissue reflected absorbed energy). In this case, we found 27 lipids (2 LPCs, 22 PCs, and 3 SMs) whose serum levels were significantly correlated with total absorbed radiation energy accumulated in the patient's body at a given time point (examples of identified compounds are presented in Table 2). Due to the characteristics of IMRT, tissue irradiated with high doses represent rather small volumes (tumor and adjacent tissues) and may have a lower impact on the systemic body's response to RT observed at the level of body fluids. Hence, this latter observation suggested the importance of "lower" doses of radiation delivered to "larger" volumes of normal tissues.

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Table 2. Examples of identified lipids which serum levels correlated with maximum GTV dose, a total absorbed radiation energy (i.e., area under DVH), or volumes of tissues irradiated at 20 or 50Gy of BED. Showed are correlation coefficients; significant correlations ($|r| > 0.3$) are marked with bold.

lipid name	m/z	max. GTV dose	total absorbed radiation energy	volume irr. at 20 Gy	volume irr. at 50 Gy
LPC(16:1)	494.33	**-0.31**	**-0.32**	**-0.33**	-0.16
LPC(18:0)	524.38	**-0.39**	**-0.37**	**-0.35**	-0.21
PC(30:0)	706.55	**-0.32**	**-0.34**	**-0.35**	-0.25
PC(30:1)	704.53	**-0.31**	**-0.34**	**-0.35**	-0.20
PC(32:2)	730.55	**-0.39**	**-0.37**	**-0.35**	-0.27
PC(34:3)	756.57	**-0.33**	**-0.35**	**-0.35**	-0.28
PC(36:2)	786.61	**-0.41**	**-0.43**	**-0.42**	-0.28
PC(36:3)	784.60	**-0.30**	**-0.30**	-0.27	-0.10
PC(38:2)	814.64	**-0.48**	**-0.51**	**-0.48**	**-0.33**
PC(38:3)	812.63	**-0.49**	**-0.50**	**-0.46**	-0.28
PC(38:5)	808.60	-0.29	**-0.34**	**-0.32**	-0.16
SM(36:0)	733.63	**0.31**	**0.37**	**0.37**	0.26
SM(36:1)	731.62	**0.34**	**0.36**	**0.36**	0.25

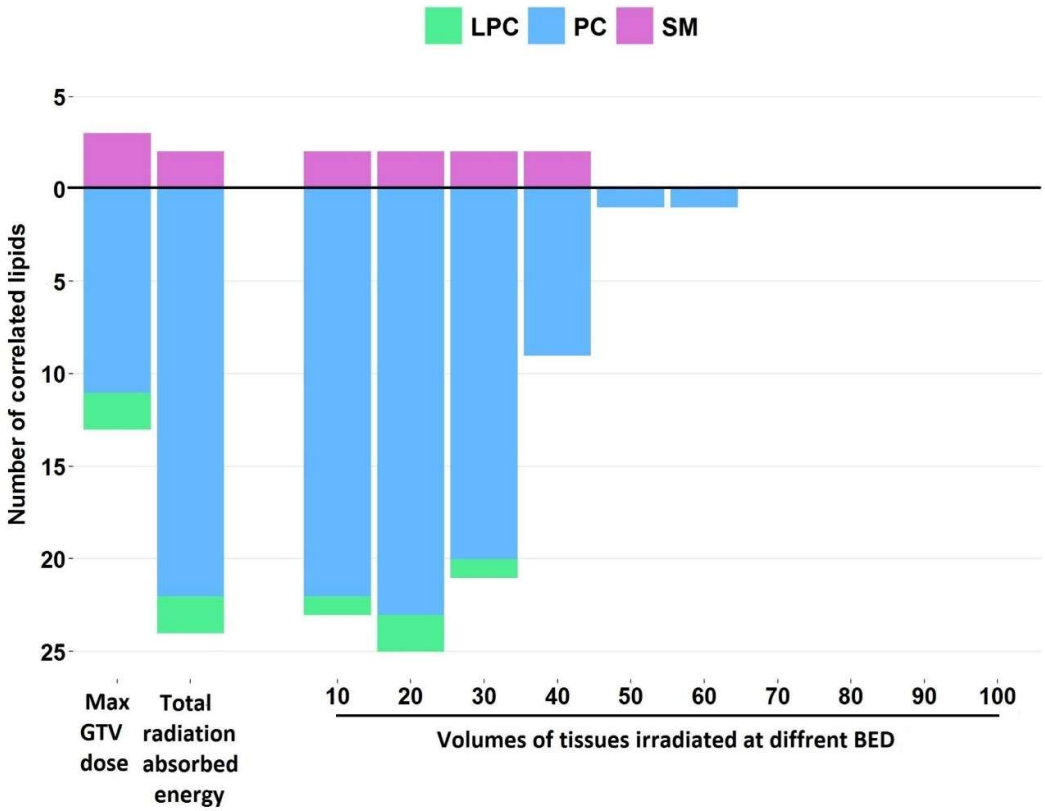

Figure 1. Numbers of lipids which serum levels were significantly correlated with maximum GTV dose, a total absorbed radiation energy (DVH area), and volumes of tissues irradiated at different BED (10 Gy intervals in the 10 to 100 Gy range). Different classes of lipids (LPC, PC, and SM) are color-coded; positive ($r > 0.3$) and negative ($r < -0.3$) correlations are presented above and below the zero line, accordingly.

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To study this effect in detail, we extracted from each DVH the irradiated tissue volumes for every 10 Gy increase of accumulated BED from 10 to 100 Gy. Then, lipids which serum levels correlated with a volume of tissues irradiated at a given BED were identified. We found the highest number of such correlations with the volume of tissues irradiated at 20 Gy BED (27 correlated compounds, examples of identified compounds are presented in Table 2). The number of compounds whose serum levels were correlated with a volume of irradiated tissues gradually decreased with a dose and practically no lipids with levels significantly correlated with volumes of tissues irradiated at BED higher than 40 Gy was observed (Figure 1).

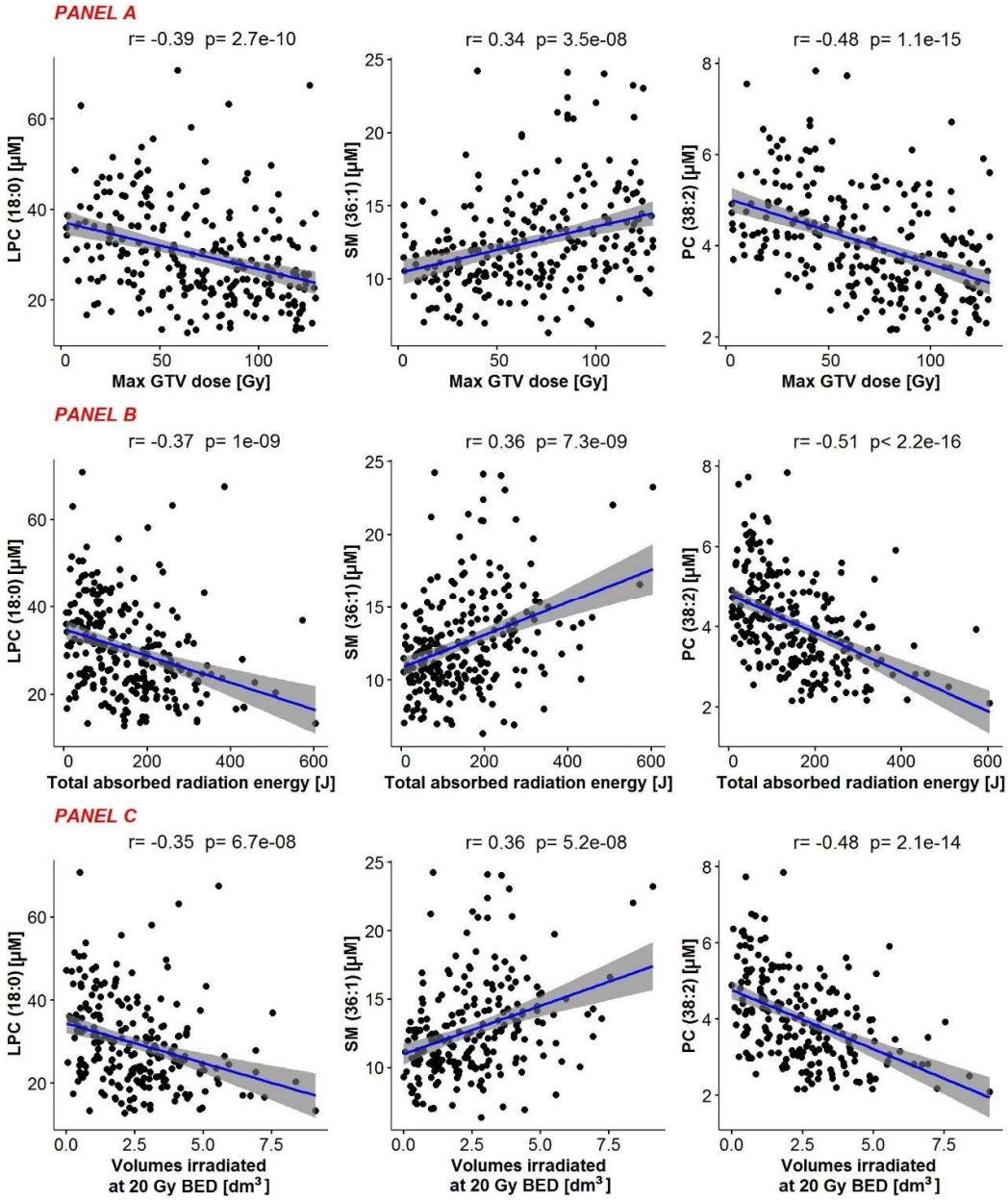

Figure 2. Correlations between serum levels of selected lipids and maximum GTV dose (Panel A), the total absorbed radiation energy (Panel B), and volume of tissues irradiated at 20 Gy BED (Panel C). Illustrated are three compounds: LPC(18:0), SM(36:1), and PC(38:2).

We found different types of correlations with dose/volume for specific types of lipids. In general, the increased dose or volume of irradiated tissues that was associated with decreased

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serum lipid level (i.e., negative correlation) was observed for several PCs and LPCs. Changes in serum levels of about a third part of detected PCs showed some correlation with radiation parameters. For example, a negative correlation was observed between levels of 25 PCs (out of 81) and volume of tissues irradiated at 20 Gy BED; this class of lipids is exemplified by PC(38:2) as illustrated in Figure 2. Similarly, negative correlations with radiation parameters were observed for 2 LPCs (out of 12 detected), namely LPC(16:1) and LPC(18:0); the latter one is illustrated in Figure 2. Noteworthy, positive correlations were observed for neither PCs nor LPCs. In marked contrast, for 3 SMs (out 51 detected) the increased serum levels were associated with increased dose or volume of irradiated (i.e., positive correlations); this class of lipids is exemplified by SM(36:1) as illustrated in Figure 2. No negative correlations with radiation parameters were observed for SMs, which indicated different modes of reactivity of SMs and PCs/LPCs.

3. Discussion

Several bioindicators of exposure to ionizing radiation have been proposed, yet the actual dose-dependence in samples of human tissues exposed to radiation *in vivo* was documented for only a few of them. Such verified "biodosimeters" include factors that could be tested in peripheral blood cells: (cyto)genetic lesions [19] and the expression of certain genes like FXDR [20]. Here we documented the dose and volume dependence of RT-induced changes in a profile of human serum phospholipids, which further extend the pool of potential biomarkers of radiation exposure. We observed a dose-dependent decrease of (lyso)phosphatidylcholines (LPCs/PCs) and increase of sphingomyelins (SMs) in the serum of patients exposed to the partial body irradiation due to head and neck cancer. Importantly, observed effects were associated not only with a maximum radiation dose delivered to the gross tumor volume but also with lower doses delivered to larger volumes of normal tissues (e.g., doses 0.5 Gy per fraction), which are frequently treated as "clinically irrelevant". We had previously found that the extent of differences in profiles of endogenous serum peptides [10] and lipids [18] between pre-RT/within-RT and post-RT samples of HNC patients treated with a continuous accelerated RT (CAIR) correlated mainly with the volume of tissues irradiated at relatively low doses (accumulated dose 10-15 Gy. Here we extended this observation for other schemes of IMRT and focused on specific classes of phospholipids.

In this work, we addressed choline-containing phospholipids present in human serum. Phosphatidylcholines, the main building blocks of membrane bilayers, are the most abundant phospholipids in serum/plasma that predominantly localize in high-density lipoproteins (HDL). Decreased levels of PCs in the serum of irradiated patients putatively reflect their rapid turnover in stressed/damaged cells and elevated uptake from the blood. In addition to their main function as a membrane constituent, PCs have a role in signaling through the generation of LPCs (by phospholipases A2), SMs (by SM synthase), phosphatidic acid (PA; by phospholipases D), and diacylglycerols (DAG; by phospholipases C) [21]. Therefore, significant downregulation of several PCs observed in serum during radiotherapy could reflect both the recovery of damaged cell membrane and increased requirements for signaling pathways that depend on PC-derived compounds. Lysophosphatidylcholines are the major bioactive component of oxidized low-density lipoproteins (LDL) mostly responsible for their inflammation-related functions [22]. Decreased levels of LPCs in blood were significantly associated with activated inflammatory status in many cancer types [23], which suggest that reduced level of LPCs observed in serum of irradiated patients could also reflect the inflammation-related aspect of radiation response. In contrast to PCs and LPCs, which serum levels decreased during radiotherapy, sphingomyelins showed radiation-related upregulation. In general, choline-based compounds are constantly transformed into each other [14]. Hence, new SM molecules are likely generated from PC compounds by SM synthase, which transfers choline "head" from PC to suitable ceramide molecule, which also explains a reduced level ceramides observed in serum of irradiated patients [18]. SMs can be hydrolyzed back to

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ceramides by SMase action. This balance between sphingomyelin production and degradation is a key factor in SM-related apoptotic signaling, and the generation of ceramides from SMs' degradation was reported to influence both the rate and the form of cell death [24]. Therefore, observed changes in serum levels of choline-containing phospholipids apparently mirror the membrane regeneration processes and signal-transduction pathways associated with treatment-induced damage of cellular and tissue components.

Interestingly, RT-downregulated LPC that is based on stearic acid (18:0) is directly produced through phospholipase A2 from PCs that contain this fatty acid. PCs that putatively contain stearic acid, namely PC(18:0/18:2), PC(18:0/18:3), PC(18:0/20:2), PC(18:0/20:3), and PC(18:0/20:5) (detected as PC 36:2, 36:3, 38:2, 38:3 and 38:5, respectively) were also downregulated by RT. Furthermore, stearic acid was a component of RT-upregulated SMs, namely SM(d18:0/18:0) and SM(d18:1/18:0) (detected as SM 36:0 and 36:1, respectively). Therefore, our observations indicate that the metabolism of stearic acid is particularly involved in radiation response. The linkage between stearic acid and choline-containing phospholipids as well as their radiation-related functions are complicated and might also be affected by other processes ongoing in the patient's body. Nevertheless, these compounds present in human serum or plasma could be used to monitor systemic effects induced by ionizing radiation with some potential as bioindicators of radiation exposure.

4. Materials and Methods

4.1. Patient Group and Material Collection.

The study involved 45 patients (all Caucasians) with locally advanced HNSCC (no distant metastases) who received RT alone and did not undergo any other treatment (surgery or chemotherapy). The patients were treated with 6 different IMRT schemes where a total dose was ranging from 51 Gy to 72 Gy, a fraction dose was ranging from 1.8 Gy to 3 Gy and a number of fractions was ranging from 17 to 40. The group of HNSCC patients was further extended and pre-RT samples of 53 patients were included. Clinical characteristics of the enrolled patients and treatment details are presented in Table 1. The study was approved by the appropriate local Ethics Committee (MSCI; approval no. 1/2016) and all participants provided informed consent indicating their conscious and voluntary participation. Blood samples were collected once a week during radiotherapy (3 to 8 samples, depending on a patient) and before the start of any treatment (53 patients). Blood samples (5 ml) were collected into BD Vacutainer Tubes and incubated for 30 min at room temperature. Next, they were centrifuged at 1000 × *g* for 10 min to remove clots. The resulting sera were portioned, then frozen, and stored at -80 °C.

4.2. Recalculation of DVH based on BED

First, we determined irradiated volumes for selected isodoses starting from 2 Gy, then every 5 Gy from 5 to 30 Gy, and every 10 Gy from 40 to 70 Gy. To account for differences in biological effectiveness between applied treatment plans isodoses received by the treated HNSCC patients were recalculated to biologically effective doses (BED). BED is a measure of the biological dose delivered by a particular combination of dose per fraction and total dose to a particular tissue characterized by a specific α/β ratio [25]. Most of the volume irradiated by IMRT was related to healthy tissue, therefore α/β ratio was kept 3 for all calculations. Isodoses from each treatment plan were first adjusted to BED and then used to determine BED-based DVH. Since DVH reflects the total dose received during the whole treatment plan, we also adjusted DVH to individual samples based on how many fractions were received by the patient before the collection of the particular sample.

4.3. Sample preparation and extraction of phospholipids

Each serum sample (10 μL) was complemented with LPC 17:1 (3.82 ng; Avanti Polar Lipids, Inc.) and PC 17:0-14:1 (52 ng; Avanti Polar Lipids, Inc.) standards before lipid extraction.

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Extraction of a lipid fraction was performed according to a modified Folch method [26]. In brief, 10 µL of serum was mixed with 350 µL of 1:1 methanol/chloroform mixture (v/v) containing antioxidants: 0.01% (w/v) 2,6-di-tert-butyl-4-methylphenol and 0.005% (w/v) retinol. Then, 100 µL of water was added. The mixture was vortexed and incubated for 30 min at 4 °C and then centrifuged (5 min, 15,000g). The chloroform phase (the bottom one) was kept and stored at -80 °C, until performing mass spectrometry analysis (within one week).

4.4. LC-MS analysis of phospholipids

The chloroform (section 4.3) was diluted ten times with acetonitrile prior LC-MS analysis. Eight µl of the resulting mixture was separated by Agilent 1290 Infinity LC (Agilent Technologies) using a 2.1× 100 mm ACQUITY UPLC BEH HILIC column (Waters) with the flow rate of 250 µL/min. The chromatography was performed using 95:5 acetonitrile/ water (solvent A) and 50:50 acetonitrile/water (solvent B), both with 10 mM ammonium acetate at pH 8.0; gradient of solvent B from 0% to 30% was applied within 15 min, and resulting fractions were analyzed online using QTOF 6540 mass spectrometer (Agilent) in the positive electrospray ionization mode. Spectra were pre-processed by peak picking and alignment, and then peak abundances (area under the peak) for each ion described by its m/z and retention time (with the integration of its isotope envelope) were estimated using the Progenesis QI data analysis software (Nonlinear Dynamics). Abundances of detected lipid ions were normalized using the LPC(17:1) and PC(17:0-14:1) standards. Cation adducts (i.e., [M + Na]+ and [M +K]+) were combined with protonated ions ([M +H]+) before further statistical analysis. To confirm lipid class and length of fatty acyl chains selected ions were analyzed by LC–MS/MS in a separate run; fragmentation patterns were verified using SimLipid software (PREMIER Biosoft).

4.5. Statistical and Bioinformatic Analyses

To detect differences between pre-RT samples of patients with more/less advanced cancer or different cancer location, we used the Mann-Whitney test (the majority of analyzed lipid levels had not normal distributions). As an adjustment for multiple testing, we used Benjamini-Hochberg correction; differences were considered significant when q-value ≤ 0.05. The area under a BED-volume curve was measured by trapezoidal rule for each sample. To calculate the volume of irradiated tissue for every 10 Gy of DVH, the spline regression model was built on the BED and radiation volume values. The knots value for BED were selected based on min value, 0.25, 0.5, and 0.75 quantiles, and max value separately for each patient. A fitted model was used to predict radiation volumes for a chosen BED value (range between 10 to 100 Gy). Spearman's correlation coefficient was computed to examine the correlation between the lipid level and max GTV dose, the area under DVH, or tissue volumes of selected BED. All analyses were performed using R statistical software package version 4.0.1. (R Foundation for Statistical Computing, http://www.r-project.org). A correlation was considered significant when its two-sided p-values ≤ 0.05 and $|r| > 0.3$ [27].

5. Conclusions

This study demonstrates the significant involvement of phospholipids based on choline head and stearic acid residue in the systemic response of the patient's body to IMRT. The serum levels of these compounds depended on the dose and volume of irradiated tissues. Higher doses and volumes were associated with reduced levels of (lyso)phosphatidylcholines and increased levels of sphingomyelins. Additionally, correlations of lipidome patterns with effects of low/moderate doses delivered to large volumes of normal tissue highlight the biological relevance of such doses during IMRT.

Author Contributions: P.W. and K.J. designed the study; K.J. an K.P. carried out the experiments; A.K. and K.J. performed the bioinformatics and statistical analysis; A.K. performed visualization; P.P., K.Sz., and K.Sk. collected and analyzed clinical data; K.Sk. provide funding; K.J. and P.W. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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